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RESPIRATION DURING MATURATION AND AT FERTILIZATION OF FISH EGGS

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A good deal of work upon this subject has been done almost exclusively on the marine invertebrate eggs, but only little on fish eggs. This may be due mainly to the fact that unfertilized eggs of fish lose their fertilizability within a few minutes when they are put into water. As to the eggs of the fresh-water fish, *Oryzias latipes*, YAMAMOTO (1939) found that the ripe unfertilized eggs are fertilizable in isotonic salt solution for a number of hours and that the fertilization process takes place quite normally in it. These findings have made it possible to study the respiratory metabolism of unfertilized eggs in the fertilizable condition. Furthermore it has become possible to measure the respiratory changes at the fertilization of *Oryzias* eggs.

In the sea urchin, *Paracentrotus lividus*, LINDAHL and HOLTER (1941) have compared the oxygen consumption of primary oocytes with that of ripe unfertilized eggs and of fertilized eggs. They found that the respiration of primary oocytes is clearly higher than that of the unfertilized eggs. Moreover, the former somewhat exceeds that of the fertilized eggs. Recently BOREI (1948), working on the eggs of *Psammechinus miliaris*, got the results somewhat different from those of LINDAHL and HOLTER. He found that the respiration of ripe unfertilized eggs declines rapidly after they have been removed from the ovary into the sea water and that its initial rate may exceed that of fertilized eggs. It was, therefore, of interest to investigate the respiratory changes of fish eggs during the maturation process. The eggs of *Oryzias* seemed particularly suitable for this purpose, because its maturation cycle is regularly repeated every 24 hours during spring and summer. But this advantage is offset by the shortage of eggs in one and the same female. Hence it is necessary to use the method which permits the measurement of the oxygen consumption of a small number of eggs. Using the Cartesian diver method it has become possible to measure the oxygen consumption of single eggs which are selected from the egg mixture. Furthermore this method has proved to be favourable for the measurement of fertilization. In mass run measurements it is inevitable that the fertilization percentage should considerably decrease, because there is necessarily some individual variability in the response to insemination. But this may be also avoided by the runs of single eggs. In addition, this paper deals with some data concerning the respiratory metabolism of unfertilized eggs.

MATERIAL AND METHOD

Materials used were eggs of an orange-red type of *Oryzias latipes*. The

general method of obtaining the eggs is the same as described by YAMAMOTO (1944). The belly of the fish was dissected out after pithing and the entire ovary was placed in the isotonic Ringer's solution of the following constituents; M/7.5 NaCl 100 parts + M/7.5 KCl 2.0 parts + M/11 $CaCl_2$ 2.1 parts, the pH of which was made 7.2 by adding phosphate buffer solution (M/100). In the same ovary different kinds of eggs are mixed. They are easily distinguishable, because ripe unfertilized eggs are more translucent than immature ones. The desired eggs were picked out under the binocular microscope and washed in the Ringer's solution. A testis was also isolated from the adult male, mashed with the blunt end of a glass rod, and diluted with the Ringer's solution. Insemination and artificial stimulation were performed in the Ringer's solution.

The diver techniques used for the measurement of oxygen consumption were essentially what were described in detail by HOLTER (1943). Some points may, however, be mentioned. The divers have volumes of about 20–30 μL, with inner neck diameters of about 1.3–1.5 mm. This rather wide neck was chosen because the diameter of fertilized eggs is about 1.2 mm. But the diffusion of gas from wide-neck diver proved to be substantially reduced with the use of a stopper in the oil seal. The divers were coated with a water-repellent silicone (NS 200) instead of paraffine wax (SCHWARTZ 1949, WATERLOW and BORROW 1949). As the physiological medium, isotonic RINGER's solution was used, and the whole process of preparing the divers was carried out in the air. Unless otherwise indicated, the diver charges were followed as in a usual manner, *viz.* the egg was placed on the bottom of a diver and the isotonic NaOH was placed in the neck. The introduction of the egg into the diver and the subsequent removal of the excess RINGER's solution was carried out, in accordance with the technical details pointed out by BOELL, NEEDHAM and ROGERS (1939). The measurements were begun less than fourty minutes after the removal of the eggs from the ovary. The oxygen consumption values were usually taken from the measurements during the first two hours. In each series of experiments at least 10 measurements were performed and from those the average values, $\bar{x}$, were calculated. In order to know the fiducial limit of the means $u\sqrt{F/N}$ was used. In this case, $\bar{x} \pm u\sqrt{F/N}$ gives the fiducial limit at the 95% fiducial probability. The temperature in the experiments was mainly 25°C. This is the moderate temperature for the normal development of *Oryzias*.

RESULTS

Respiration during maturation

Since the adult females of *Oryzias* lay eggs daily for a considerable period during spring and summer, the maturation cycle proceeds almost invariably. From the ovary it was accordingly possible to pick out different kinds of oocytes for the diver experiments. The oocytes were tentatively classified into three groups according to size; small, medium and large oocytes. They were taken every 12 hours before spawning. As is well known, oocytes are invested by follicle cells which are surrounded by a much less distinct layer of cells. The stickiness of these cells made it difficult to remove the follicular wall from the surface of smaller oocytes. Therefore, the diver measurements were made on

the oocytes with follicular tissues. The diameter of oocytes, including the follicular walls, was measured, and from this the volume was calculated, assuming the shape of the oocyte as a sphere. As shown in Table 1 the volume of oocyte varies two- to fourfold within 24 hours. The oxygen consumption figures obtained from these oocytes are given in Table 2. It will be seen that the rate of the oxygen consumption increased rapidly during the growth of oocyte and there is a close correlation with the size. The increasing oxygen consumption following the growth of oocyte would be indicative of the intense metabolism associated with the yolk formation. It was also noticed that at any stages the oocyte absorbs about 0.2 μL oxygen per μL of oocyte volume and hour. Since the inert part for respiration may increase on the advancing yolk formation, it is plausible that the respiratory activity of oocyte substantially increases during its growth. These rates of oxygen consumption, however, are accompanied by that of follicle tissues. It is probable that the observed increase is due partly to the follicle cells. In order to elucidate this problem, the following experiments were made.

Table 1. Diameter and volume of the oocytes of *Oryzias latipes* during growth. Each figure represents the average value based on 20 oocytes.

Oocyte type	Time before spawning (hours)	Diameter (mm.)		Volume (μL)
		$\bar{x}$	$u\sqrt{F/N}$	
Small-sized oocyte	36	0.76	0.063	0.23
Medium-sized oocyte	24	0.97	0.096	0.49
Large oocyte	12	1.12	0.026	0.73

Table 2. Average rate of the oxygen consumption of *Oryzias* oocytes during growth.

Oocyte type	Oxygen consumption (μL/egg/hour)	
	$\bar{x}$	$u\sqrt{F/N}$
Small-sized oocyte	47.1×10^{-3}	3.8×10^{-3}
Medium-sized oocyte	87.5×10^{-3}	4.0×10^{-3}
Large oocyte	171.9×10^{-3}	8.5×10^{-3}

When the oocyte reaches its maximum size, *viz.* large oocyte, it is possible to remove the follicular wall by means of two fine forceps and to clean the denuded oocyte from the adhering follicle cells by washing in isotonic KCl solution. This denuded oocyte appears exactly as it does in the follicle sac except for a slight swelling in the isotonic Ringer's solution. The results from the measurements of this denuded oocyte are shown in Table 3. Comparing the respiration value of the denuded oocyte (Table 3) with that of the entire oocyte (Table 2), we found that the respiration of oocyte proper is about two-thirds of that of the entire oocyte which includes the follicle tissue. Nevertheless, the former has higher metabolism than the medium-sized oocyte. This indicates that the true respiratory rate of oocyte also increases during the growth phase

of oocyte. In the large oocyte, the nucleus is still in the germinal vesicle stage and numerous oil droplets are scattered beneath the egg membrane (Fig. 1).

Table 3. Average rate of the oxygen consumption of the eggs of *Oryzias latipes* during maturation.

Stage of maturation	Oxygen consumption (μL/egg/hour)		Fertilizability
	$\bar{x}$	$u\sqrt{\bar{F}/\bar{N}}$	
Large oocyte	112.0×10^{-3}	14.3×10^{-3}	non-fertilizable
Uuder-ripe egg	41.9×10^{-3}	3.4×10^{-3}	non-fertilizable
Ripe unfertilized egg	24.3×10^{-3}	1.6×10^{-3}	fertilizable

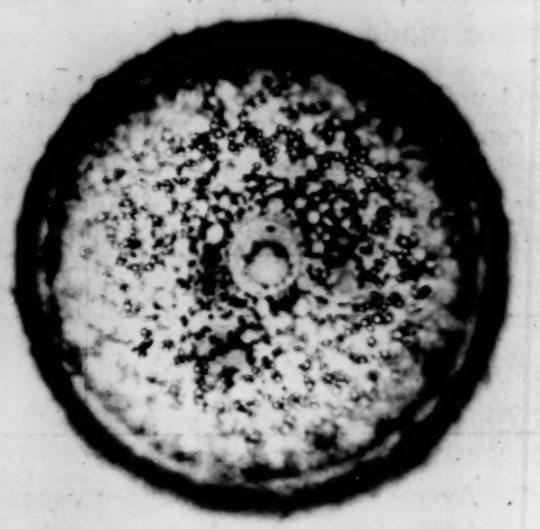

Figure 1. The large oocyte of *Oryzias latipes.*

Later on the maturation division begins and the germinal vesicle breaks down. If these oocytes are excised and placed in the RINGER's solution, the rupture of the follicle will occur within a short time in some of them. After this, the egg will be seen to emerge from the follicle. In this case the partial emergence of the egg often takes place, perhaps because of the slight contraction of follicles as described by RUGH (1935) in the frog egg. Artificial rupture with fine forceps can be made easily in this stage. But the eggs are still unfertilizable even if their follicular walls have been removed. This may be due to the fact that cytoplasmic maturity is not reached until considerably later. These eggs are termed under-ripe eggs (Fig. 2).

As the maturation proceeds, the oil droplets decrease in number by confluence and the cortical alveoli are clearly seen in the cortical layer of the eggs. Such eggs are almost all ovulated in the Ringer's solution and are fertilizable (Fig. 3). Under the normal condition, ovulation takes place in the middle of night as described by ROBISON and RUGH (1943). Then the eggs are held in the

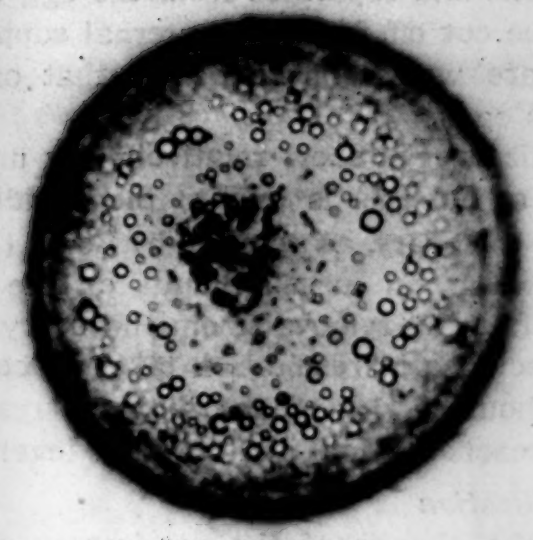

Figure 2. The under-ripe egg of *Oryzias latipes* which shows the breakdown of the germinal vesicle.

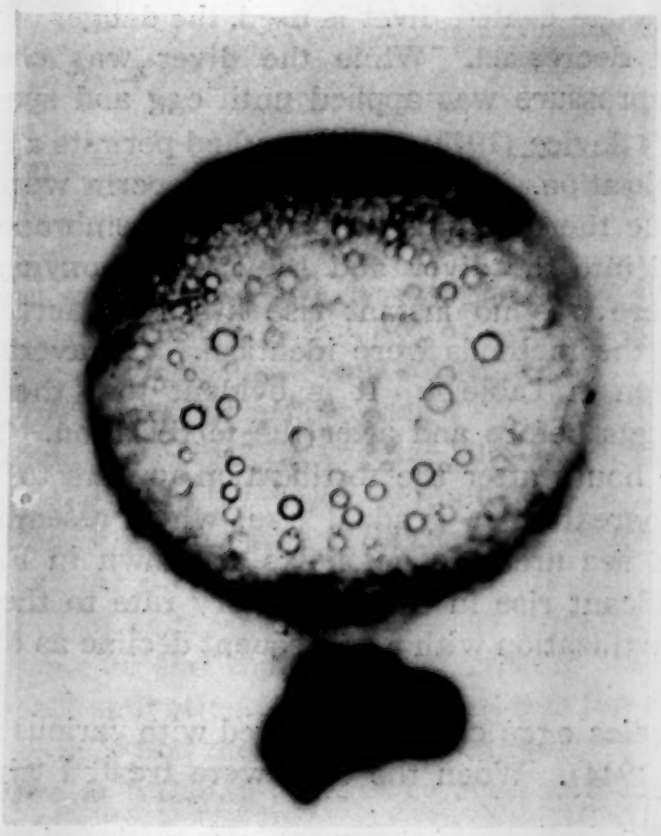

Figure 3. The ripe unfertilized egg of *Oryzias latipes*, just ovulated in the Ringer's solution.

ovarian lumen until all the matulated ones have ovulated and oviposition occurs in the early morning. The average oxygen consumption of the eggs during maturation is tabulated in Table 3. It will be seen that the respiration of the oocytes gradually decreases as the process of maturation advances. This decrease would be accompanied by a decrease in the amount of substrates which are

supplied through the follicles. During the maturation division follicular wall becomes more transparent and separates from the egg surface. This suggests that the oocyte might be cut off from the external supply of substrates in this stage. These results are well consistent with that of LINDAHL and HOLTER (1941) on *Paracentrotus* eggs.

As the ripe unfertilized eggs show relatively low metabolism, the question may be asked whether or not this is due to the rapidly declining respiration after the removal from the ovary, as reported by BOREI (1948) on the unfertilized eggs of *Psammechinus*. Careful measurements on the unfertilized eggs were repeated immediately after the removal from the ovary and were continued for some hours. But no decrease in respiration takes place and the linear course is maintained for four hours. This means that the respiratory metabolism of ripe eggs has already reached a low and constant level in the ovarian lumen.

Respiration at fertilization

In order to study the change of the respiratory rate at fertilization, the divers were charged as follows, according to the "Diver charge Type II" in BOREI (1948) : the unfertilized egg was placed in the neck, a side drop of sperm was placed in the lower end of the neck and alkali solution at the bottom of the diver. If the silicone coated diver is used, the danger of premature fertilization is considerably decreased. While the diver was observed through the microscope, the overpressure was applied until egg and sperm were mixed by the use of the CLAFF's device (1949). This method permits a continuous measurement following fertilization. In some cases, the sperm were added to the unfertilized eggs outside the diver. As soon as the membrane was separated, the eggs was introduced into the diver and the rate of oxygen consumption was measured. Since there was no instant rise after the fertilization, the results obtained by both of the methods were identical. The average values of oxygen consumption are given in Table 4. It is evident that there is no perceptible change in *Oryzias* eggs before and after the fertilization. The respiratory rate within two or three hours after the fertilization appears to have a more or less constant value. Thereafter a gradual rise, which resembles the exponential increase in fertilized sea urchin eggs, starts as shown in Fig. 4. There is no evidence for a significant rise in the respiratory rate to the maximum about 90 minutes after the fertilization with a subsequent decline as expected from BOYD's results (1928).

Unfertilized *Oryzias* eggs can be activated with various physical or chemical agents (YAMAMOTO 1944). When the eggs were treated with these agents, the

Table 4. Comparison of the oxygen consumption of the eggs of *Oryzias latipes* before and after fertilization.

	Oxygen consumption (μL/egg/hour)	
	$\bar{x}$	$u\sqrt{F/N}$
Unfertilized egg	24.3×10^{-3}	1.6×10^{-3}
Fertilized egg	24.5×10^{-3}	1.6×10^{-3}
Activated egg	24.6×10^{-3}	2.4×10^{-3}

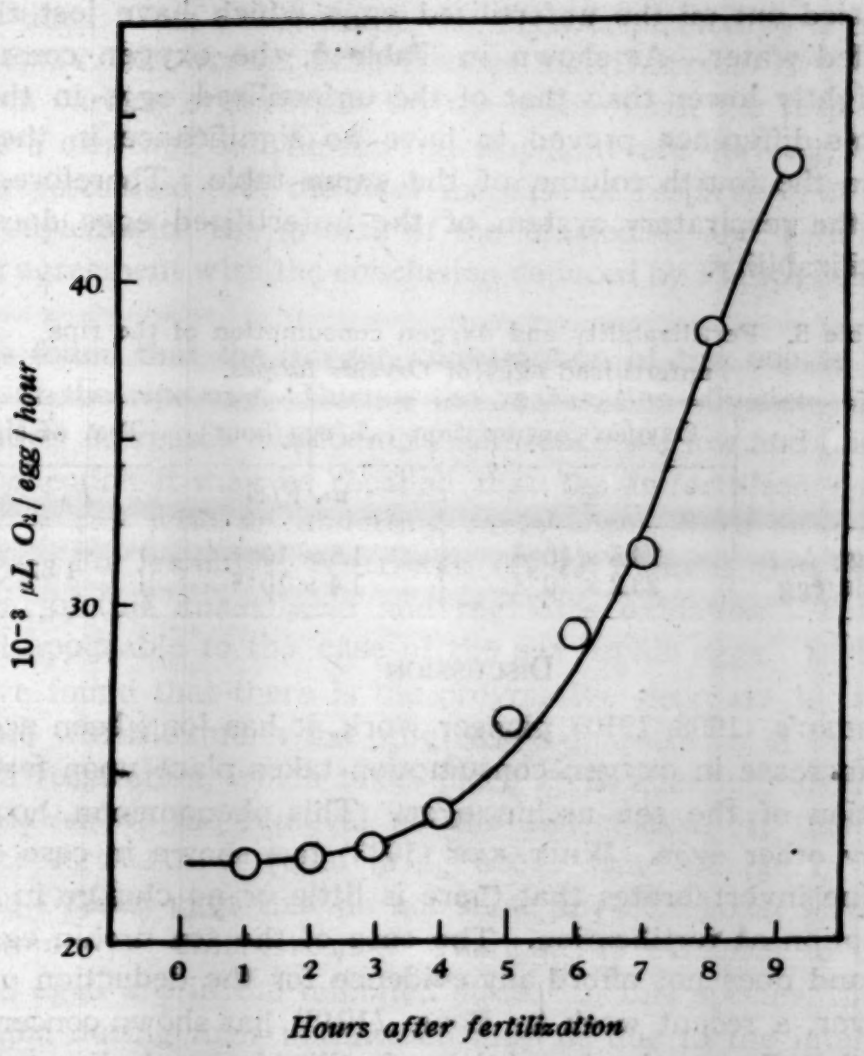

Figure 4. Oxygen consumption of the *Oryzias* eggs during development.

breakdown of the cortical alveoli and the subsequent elevation of the egg membrane take place. Afterwards the blastodisk is formed at the animal pole and oil droplets are accumulated at the vegetal pole as in the normal fertilization process. Most of the eggs, however, remain at the one-cell stage and show no further development. It seemed interesting to measure the oxygen consumption of these activated eggs and to compare it with the respiration of normal fertilized eggs. The eggs were activated by pricking with a fine glass needle of 10-20 μ in diameter and the activation process was observed. After several minutes, a perfect-activating egg was picked out for the run. This egg was introduced into the diver and the rate of oxygen consumption was measured. The results are tabulated in Table 4. It will be seen that there is no difference between the respiration of fertilized eggs and that of activated eggs. However, prolonged experiment showed that the oxygen consumption of activated eggs remained at the constant level, in contrast with the results of the fertilized eggs.

As stated above, the unfertilized eggs of fish lose their fertilizability within a few minutes when they are put into water. In *Oryzias* eggs, this capacity is lost in tap water after six minutes (YAMAMOTO 1944). The question may arise as to the fertilizability and the metabolic systems of the eggs. Measure-

ments were carried out on the unfertilized eggs which have lost their fertilizability in distilled water. As shown in Table 5, the oxygen consumption of these eggs is slightly lower than that of the unfertilized eggs in the RINGER's solution. But this difference proved to have no significance in the statistical test, as shown in the fourth column of the same table. Therefore, it may be postulated that the respiratory system of the unfertilized eggs does not alter after losing fertilizability.

Table 5. Fertilizability and oxygen consumption of the ripe unfertilized eggs of *Oryzias latipes*.

	Oxygen consumption (μL/egg/hour)		Test of significance	
	$\bar{x}$	$u\sqrt{F/N}$	F_0	F 5%
Fertilizable egg	24.8×10^{-3}	1.5×10^{-3}	$1.32 < 4.35$	
Non-fertilizable egg	23.2×10^{-3}	1.4×10^{-3}		

DISCUSSION

Since WARBURG's (1908, 1910) pioneer work, it has long been accepted that a considerable increase in oxygen consumption takes place upon fertilization or artificial activation of the sea urchin eggs. This phenomenon, however, does not apply to any other eggs. WHITAKER (1933) has shown in case of the eggs of various marine invertebrates that there is little or no change in the rate of oxygen consumption at fertilization. The case of the sea urchin eggs seems to be special one and does not afford any evidence for the deduction of a general nature. Moreover, a recent work by BOREI (1948) has shown concerning *Psammechinus* eggs that the respiration of the unfertilized eggs declines rapidly after they have been removed from the ovary into the sea water. This means that, if fertilization occurs rather soon after the removal of the eggs from the ovary, the respiration does not change before and after fertilization.

In the fish eggs there are only a few studies on the change of the respiration at the time of fertilization. BOYD (1928) found in *Fundulus* eggs that there was a marked rise in the oxygen consumption after fertilization. On the contrary, PHILIPS (1940) has stated that such a transient rise in the respiration did not take place after fertilization. In these previous investigations the eggs have been suspended in the normal sea water, in which the unfertilized eggs of *Fundulus* lose their fertilizability within 15–20 minutes (KAGAN 1935). Therefore, it would be pointless to try to compare the respiratory metabolism before and after fertilization. In the eggs of *Oryzias* this difficult is overcome when the measurements were carried out on the unfertilized eggs suspended in the isotonic RINGER's solution. From the measurements in this manner, it was found that no immediate rise in respiration takes place at fertilization. Similar results were obtained with the artificially activated eggs. In both of the cases the cortical change takes place and the subsequent elevation of the egg membrane ensues. These facts indicate that the cortical change does not alter the respiratory activity of the eggs.

The respiration of fertilized eggs increases gradually as an exponential func-

tion with time. This phenomenon has been confirmed by a number of workers (notably GRAY 1926, ATLAS 1938, FISCHER and HARTWIG 1938 and LINDAHL 1939), working on various organisms. On the other hand, the respiration of activated eggs, where cleavage and further development are lacking, does not increase. It may be postulated that the later increase of respiration of the fertilized eggs may be coupled with the growth of the blastodisk and cellular multiplication. This is in agreement with the conclusion deduced by Philips concerning *Fundulus* eggs.

It was found that the oxygen consumption of the oocyte is clearly higher than that of the ripe egg. During the maturation division, however, the rate of respiration decreases considerably and reaches a low and fairly constant level. In this connection it may be recalled that the unfertilized egg has been considered as a cell with an abnormal metabolism, fixed in a state resembling anaesthesia. For example, WHITAKER (1933) thought that fertilization brings the egg out of this anaesthesia and regulates oxidation. This concept seems to be well applicable to the case of the sea urchin eggs. LINDAHL and HOLTER (1941) have found that there is the progressive decrease in respiration during maturation; which exhibits the progressive anaesthesia of the egg. A marked increase in respiration, which takes place at fertilization, should be considered as an indication of the removal of this anaesthesia. If this interpretation is correct, the fertilization would bring about some change in respiration. The case of the *Oryzias* eggs that do not show any change at fertilization remains, however, somewhat embarrassing. There is no evidence to consider that the unfertilized eggs are in the inhibited state. In this species, the rapid decrease of respiration during their maturation may be due to the interception from the external supply of substrates by follicle cells. Further it can be seen that cyanide strongly inhibits the oxygen consumption of unfertilized eggs (*cf.* ISHIDA 1948). This is in accordance with the recent researches of ROBBIE (1946) on the sea urchin eggs, in the sense that there are no qualitative differences between the respiratory system of the unfertilized eggs and the fertilized ones. However it is certain that the metabolism of fish and sea urchin eggs is perhaps different. The respiration of the unfertilized eggs of *Oryzias* does not show any decline after the removal from the ovary, in contrast with the results obtained by BOREI (1948) on the sea urchin eggs. It is of interest to note that their respiratory activity remains invariable after losing fertilizability in distilled water. These facts suggest that, in the unfertilized eggs of *Oryzias*, the respiratory system may be rather stable.

In the growth phase of oocyte, the respiratory activity rapidly increases within a short time. This may well be associated with an intense yolk formation. MESTCHERSKAJA (1935) has shown in the frog oocytes that the respiration is particularly high when the yolk formation begins and then falls after the accumulation of yolk. In the oocytes of *Oryzias*, the drop in the respiration was not found in the oocyte stage, but was found after the beginning of the maturation division. This may be due to the fact that the maturation division takes place immediately after the completion of the growth of oocyte.

Summary

1. By using the Cartesian diver method the oxygen consumption of oocytes, unfertilized eggs and fertilized eggs from the fresh-water fish, *Oryzias latipes*, was measured.

2. The respiration of oocytes increases rapidly as cell growth advances. The intimate correlation was found between the rate of oxygen consumption and the volume of oocytes.

3. The respiratory rate of oocytes is clearly higher than that of the ripe unfertilized eggs. As maturation division proceeds, the respiration decreases gradually and reaches a low and constant level. There is no declining respiration in the unfertilized eggs after their removal from the ovary.

4. No instant rise in oxygen consumption was observed at fertilization, but there is a gradual rise, following an exponential function with time. The respiratory activity of the unfertilized eggs does not change when their fertilizability has been lost in distilled water.

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